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N-truncation and pyroglutaminylation enhances the opsonizing capacity of Aβ-peptides and facilitates phagocytosis by macrophages and microglia

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ABSTRACT

Abnormal accumulations of amyloid-β (Aβ)-peptides are one of the pathological hallmarks of Alzheimer's disease (AD). The precursor of the Aβ-peptides, the amyloid precursor protein (APP), is also found in peripheral blood cells, but its function in these cells remains elusive. We previously observed that mononuclear phagocytes release Aβ-peptides during activation and phagocytosis, suggesting a physiologic role in inflammatory processes.

Here, we show that supplementing the media with soluble N-terminally truncated $Aβ_{(2–40)}$ and $Aβ_{(2–42)}$ as well as $Aβ_{(1–42)}$ induced the phagocytosis of polystyrene particles (PSPs) by primary human monocytes. If the PSPs were pre-incubated with Aβ-peptides, phagocytosis was induced by all tested Aβ-peptide species. N-terminally truncated $Aβ_{(x–42)}$ induced the phagocytosis of PSPs significantly more effectively than did $Aβ_{(x–40)}$. Similarly, the phagocytosis of *Escherichia coli* by GM-CSF- and M-CSF-elicited macrophages as well as microglia was particularly facilitated by pre-incubation with N-terminally truncated $Aβ_{(x–42)}$. The proinflammatory polarization of monocytes was indicated by the reduced MSRI expression and IL-10 secretion after phagocytosis of PSPs coated with $Aβ_{(1–42)}$, $Aβ_{(2–42)}$ and $Aβ_{(3p–42)}$. Polarization of the macrophages by GM-CSF reduced the phagocytic activity, but it did not affect the capabilities of Aβ-peptides to opsonize prey.

Taken together, Aβ-peptides support phagocytosis as soluble factors and act as opsonins. Differential effects among the Aβ-peptide variants point to distinct mechanisms of interaction among monocytes/macrophages, prey and Aβ-peptides. A proinflammatory polarization induced by the phagocytosis of Aβ-peptide coated particles may provide a model for the chronic inflammatory reaction and sustained plaque deposition in AD.

1. Background

Amyloid-β (Aβ)-peptides, the primary components of neuritic plaques found in brains of Alzheimer's disease (AD), are generated by the proteolytic processing of the β-amyloid precursor protein (APP) (Selkoe, 2011; De Strooper et al., 2012). Beneath the β- and γ-secretases, several other proteases, such as meprin-β, caspase and aminopeptidase A, seem to be involved in this process (Takeda et al., 2004; Sevalle et al., 2009; Bien et al., 2012). Thereby more than 40 different N- and C-terminally truncated and modified variants of the Aβ-peptides are generated (Maler et al., 2007).

APP is also present in the immune cells of the central nervous system (CNS) and the periphery, particularly microglia and

Abbreviations: Aβ, amyloid-β; AD, Alzheimer's disease; APP, amyloid precursor protein; BSA, bovine serum albumin; CNS, central nervous system; DAMP, damage-associated molecular pattern; DAPI, 4′,6-diamidino-2-phenylindole; *E. coli*, *Escherichia coli*; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; iNOS, inducible nitric oxide synthase; MACS, magnetic-assisted cell sorting; MFI, mean fluorescent intensity; MSRI, macrophage scavenger receptor I; OpsR, opsonizing reagent; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; PMA, phorbol-12-myristate-13-acetate; PRR, pattern recognition receptor; PSP, polystyrene particle; rhGM-CSF, recombinant human granulocyte-monocyte-colony stimulating factor; rhM-CSF, recombinant human monocyte-colony stimulating factor; sAPPα, soluble amyloid precursor protein α; sAPPβ, soluble amyloid precursor protein β; TLR, toll-like receptor.

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monocytes (Ledoux, 1993; Bitting et al., 1996; Jung et al., 1999; Spitzer et al., 2010). The induction of APP and Aβ-peptide secretion in activated mononuclear phagocytes suggests a role for APP in the initiation of immune responses (Monning et al., 1990; Sondag and Combs, 2004).

Both, the expression of surface receptors and cytokine secretion by macrophages and microglia are context sensitive. Thus, proinflammatory M1- and anti-inflammatory M2-polarized mononuclear phagocytes represent the extremes of a heterogeneous continuum (Mantovani et al., 2004; Varnum and Ikezu, 2012). Although helpful as a model for investigating the basic functions of mononuclear phagocytes, recent research has identified several intermediate stages and cells that express M1 and M2 markers simultaneously (Xue et al., 2014).

In brain sections from AD patients, microglia predominately presented markers of M1 polarization (Michelucci et al., 2009; Varnum and Ikezu, 2012; Sudduth et al., 2013). The proinflammatory polarization of microglia was shown to inhibit the clearance of Aβ-peptides and might therefore favor the accumulation of Aβ-peptides and consequent neuronal cell death, finally leading to cognitive deterioration and behavioral disturbances (Yamamoto, 2008).

Several studies have investigated the phagocytosis of Aβ-peptides as a means to eliminate them from the organism, but data on a potential physiological role for Aβ-peptides in the process of phagocytosis are scarce. Reduced levels of Aβ-peptides in CSF are found not only in AD but also in several other neuroinflammatory diseases, such as borreliosis, herpes encephalitis and bacterial meningitis, with normalization after successful treatment (Sjogren et al., 2001; Krut et al., 2013). Similarly, the CSF levels of the soluble APP fragments sAPPα and sAPPβ are reduced in multiple sclerosis and Lyme neuroborreliosis (Mattsson et al., 2009, 2010). Furthermore, systemic bacterial infections are considered to be a risk factor for sporadic AD, connecting infection, inflammation and alterations in amyloid metabolism and leading to cognitive disturbances (Dunn et al., 2005; Honjo et al., 2009; Eikelenboom et al., 2012).

A potential function of the Aβ-peptides in this context may be to opsonize pathogens to support their clearance and/or act as soluble factors with cytokine or chemokine activities. We have previously reported that monocyte activation by the phagocytosis of polystyrene beads induces APP glycosylation and Aβ-peptide secretion (Spitzer et al., 2010). We observed a relative increase in the release of N-terminally truncated Aβ peptide species from activated monocytes (Maler et al., 2008; Spitzer et al., 2010). In the current study, we used mononuclear phagocytes as a model to investigate the impact of various Aβ-peptides on the phagocytosis of polystyrene particles or *Escherichia coli* bacteria and on concurrent macrophage polarization.

2. Methods

2.1. Cell isolation and culture

Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll (Biochrom, Germany) density gradient centrifugation from buffy coats purchased at Transfusionsmedizin Suhl, Germany. Thrombocytes were removed by under-laying an FCS-gradient and centrifugation at 75*g* for 15 min. Monocytes were isolated from PBMCs by the antibody-mediated removal of non-monocytes using a MACS Monocyte Isolation Kit II (Miltenyi Biotec, Germany) and MACS LS Columns (Miltenyi Biotec, Germany). For the analysis of undifferentiated monocytes, the cells were resuspended in serum-free AIM-V medium and seeded at a density of 1×10^6 cells/ml in 96-well ultra-low attachment plates (Corning, USA). Differentiated macrophages were obtained by cultivating monocytes for seven days at 8×10^5 cells/ml in RPMI 1640 with 10% FCS in 96-well plates (Biochrom, Germany). For the polarization of macrophages, 40 ng/ml rhGM-CSF (Immunotools, Germany) or 80 ng/ml rhM-CSF (Immunotools, Germany) was added to the culture medium, respectively. After three days, 50% of the medium was exchanged.

THP-1 cells were obtained from ATCC and maintained below 1×10^6 cells/ml in RPMI 1640 supplemented with 10% FCS. For differentiation to macrophages, THP-1 cells were cultured at 2×10^6 cells/ml for three days in RPMI 1640 medium supplemented with 10% FCS and 50 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma–Aldrich, Germany).

Primary cultures of porcine microglia were isolated following a protocol adapted from Franke (Franke et al., 2000). Briefly, the secretory areas, cerebellum and meninges were removed from brain hemispheres obtained from a local abattoir. After mincing the tissue, it was incubated for 2 h with 6.5% (v/v) dispase (BD, Germany) at 37 °C. Lipids were removed by mixing 100 mL of the cell suspension with 150 mL of dextran solution (ρ = 1.0621 g/mL), followed by centrifugation at 6800*g* for 10 min at 4 °C. The tissue was further homogenized by filtration (180 μm), trituration and consecutive incubation for 30 min with 1 mg/mL collagenase/dispase (Roche, Germany). The cell suspension was layered onto a two-level percoll gradient with ρ = 1.08 and ρ = 1.04 g/mL. Mixed brain cells were collected from the lower interface of the gradient and were washed and seeded in Dulbecco's modified eagle's medium, supplemented with 10% fetal calf serum and antibiotics. Microglia were collected after 7 days by gently washing the confluent cell layer and collecting the loosely adherent cells. Finally, the microglia were plated in RPMI medium supplemented with 10% fetal calf serum and antibiotics at a density of 0.8×10^6/mL in 96-well plates.

2.2. Staining of inducible nitric oxide synthase (iNOS) in macrophages

After seven days *in vitro*, macrophages were detached with Accutase® (PAA, Germany) supplemented with 2 mmol EDTA for 45 min at 37 °C and fixed with 2% paraformaldehyde on poly-l-lysine-coated slides for 60 min at room temperature. Subsequently, the cells were permeabilized and blocked in PBS with 1% bovine serum albumin (BSA)/5% goat serum/0.2% Triton-X-100 for 1 h at room temperature. Labeling with mouse anti-human iNOS monoclonal antibody (R&D Systems, USA) was performed at a concentration of 20 μg/ml for 80 min at room temperature followed by staining with secondary antibody AF488 goat anti-mouse (Invitrogen, Germany) for 1 h at room temperature. Slides were mounted with Roti®-Mount FluorCare DAPI (Roth, Germany), and images were acquired on a Nikon eclipse 80i microscope equipped with NIS-elements BR 3.1 software.

2.3. Coating of polystyrene particles and E. coli bacteria with Aβ-peptides

$Aβ_{(1–40)}$, $Aβ_{(1–42)}$, $Aβ_{(2–40)}$, $Aβ_{(2–42)}$, $Aβ_{(3p–42)}$ and $Aβ_{(5–42)}$ (all Anaspec, USA) were reconstituted in 1% NH_4OH, diluted with H_2O_{dd} to reach a final concentration of 1 mg/ml in H_2O_{dd}/0.08% NH_4-OH and stored in aliquots at −20 °C. Yellowgreen Flouresbrite® (Polysciences, Germany) polystyrene particles (PSP) with a diameter of 1 μm were resuspended at 4.55×10^{10} particles/ml in the respective Aβ-peptide solution for 12 h at 37 °C. After washing, the particles were centrifuged at 10,000*g* for 10 min and suspended in PBS. For the phagocytosis assay, the particles were diluted in the appropriate cell culture medium to reach a final concentration of 1.5×10^8 particles/ml. The coating of PSP with bovine serum albumin (BSA, Sigma, Germany) was performed equivalently.

The AF488-labeled *E. coli* BioParticles® (Invitrogen, Germany) were reconstituted at 20 mg/ml in H_2O_{dd} with 2 mM sodium azide and coated with the respective Aβ-peptides, BSA or opsonizing reagent (OpsR, Invitrogen, Germany) as described above. The *E. coli* were diluted in cell culture medium to reach a final concentration of 0.8×10^8 particles/ml.pHrodo Green-labeled *E. coli* BioParticles (Invitrogen, Germany) were reconstituted at 2 mg/mL in PBS and were treated equivalently.

The amount of Aβ-peptide bound to the polysterene particles was assessed by staining with Aβ-peptide-specific antibodies and measurement by flow cytometry. Therefore, 4.55×10^6 particles in 100 µl PBS were incubated with 1 µg of the Aβ-peptide-specific mouse anti-human monoclonal antibody 6E10 or 4G8 (Covance, USA) for 30 min at 4 °C. The particles were washed in PBS and incubated with an AF-488-labeled secondary antibody (Invitrogen, Germany) for 30 min at 4 °C. After washing with PBS, the particles were suspended in Jonosteril® (Fresenius Kabi, Germany) for flow cytometric analysis.

2.4. Quantification by flow cytometry of in vitro phagocytosis

Immediately before the experiments, the medium was exchanged and the cells were pre-incubated for 2 h with the Aβ-peptides (1 µg/ml) or 5 µM cytochalasin D where indicated. Then, PSPs or AF488 *E. coli* were added at a final concentration of 4.65×10^6 particles/ml or 5×10^6 particles/ml, respectively. pHrodo Green-labeled *E. coli* were added at a concentration of 50 µg/mL. Opsonizing reagent was used as a positive control when the phagocytosis of AF488 *E. coli* was assessed.

For the phagocytosis of PSPs and *E. coli* particles, monocytes, THP macrophages, monocyte-derived macrophages and porcine microglia were incubated at 37 °C for 20 h, 4 h, 2 h and 4 h, respectively. THP macrophages were detached with 2.5% trypsin for 30 min. Accutase® supplemented with 2 mmol EDTA was used for the detachment of monocyte-derived macrophages and microglia. Phagocytosis was evaluated by the mean fluorescence intensity (MFI) of phagocytes as a measure of the number of cell-associated fluorescent PSPs. The non-specific binding of the beads to the cell membrane was assessed by pretreating the culture for 2 h with 5 µM cytochalasin D.

2.5. Differentiation of monocytes with Aβ-peptide-coated PSP

The isolation of human monocytes was performed as described above. The cells were cultured in 24-well plates (Biochrom, Germany) for three days at a density of 1.2×10^6 cells/ml in RPMI 1640 medium supplemented with 10% FCS. The coating of non-fluorescent PSP with a diameter of 1 µm (Micromod, Germany) with Aβ-peptides and BSA was performed as described above. PSPs were added to the cell cultures at a final concentration of 1.24×10^7 particles/ml for 72 h.

2.6. Expression of surface antigens on macrophages

A total of 1×10^5 detached cells were incubated with fluorescence-labeled monoclonal mouse anti-human MSRI-pe (R&D Systems, USA), IL1-RI-pe, IL1-RII-fitc (BD Pharmingen, Germany), HLA-DR-fitc, CD11b-fitc, CD14-pe (Immunotools, Germany) and CD 206-fitc (R&D Systems, USA) or with an appropriate isotype control for 30 min at 4 °C. Following incubation, samples were diluted with Jonosteril® (Fresenius Kabi, Germany) and measured on a CyFlow space (Partec, Germany) using the FlowMax 2.81 software.

2.7. Measurement of IL-10 and TNFα production

After 72 h of monocyte cultivation with PSP, the supernatants were harvested and stored at −20 °C until further analysis. The IL-10 and TNFα levels were measured using the DuoSet® ELISA kit (R&D Systems, USA) according to the manufacturer's instructions.

2.8. Statistical analysis

Statistical analysis was performed using the GraphPad Prism® 6.0 software. All independent experiments were repeated at least four times. The data are expressed as the mean ± SD. Significant differences were evaluated using ANOVA followed by the Neuman–Keuls post test. To detect the differences in macrophage differentiation, ANOVA for paired samples was used, followed by Fisher's least significant different test. Correlations were evaluated by Spearman's test. The criterion of significance was set to $p < 0.05$.

3. Results

3.1. Soluble Aβ-peptides enhanced the phagocytosis of PSPs by human monocytes

To investigate the effect of soluble Aβ-peptides on the phagocytosis of PSPs by freshly isolated human monocytes, the cells were pre-incubated with 1 µg/ml of the respective Aβ-peptide in cell culture medium. Then, 20 h after adding the fluorescent PSPs, phagocytosis was quantified by flow cytometry. The MFI of the phagocytes was used as a measure of the number of internalized fluorescent particles. The pre-incubation of monocytes with $Aβ_{(1–42)}$ as well as the N-terminally truncated $Aβ_{(2–40)}$ and $Aβ_{(2–42)}$, but not $Aβ_{(1–40)}$, induced phagocytosis at levels significantly above the control levels ($p < 0.05$) (Fig. 1A). Monocytes treated with $Aβ_{(2–40)}$ internalized 17% more PSPs than those treated with full-length $Aβ_{(1–40)}$ ($p < 0.05$). The treatment of cells with BSA did not influence the phagocytosis of PSPs.

3.2. Pre-incubation with N-truncated $Aβ_{(2–42)}$ increased the phagocytosis of PSPs by human monocytes most efficiently

To assess whether the Aβ-peptides secreted by monocytes enhanced phagocytosis by binding to pathogens, the effect of Aβ-coated PSPs on their phagocytosis by human monocytes was examined. The phagocytosis of fluorescent particles was quantified by flow cytometry as described above (Fig. 1B). Precoating the fluorescent PSPs with all of the tested Aβ-peptides increased their phagocytosis by monocytes compared to the phagocytosis of uncoated PSP ($p < 0.001$). Coating the PSPs with $Aβ_{(1–42)}$ enhanced the amount of phagocytosed PSPs by 40% ($p < 0.0001$). $Aβ_{(1–42)}$ induced phagocytosis more effectively than $Aβ_{(1–40)}$ ($p < 0.0001$). The treatment of monocytes with $Aβ_{(2–42)}$− and $Aβ_{(3p–42)}$-coated PSPs resulted in an even higher increase of the MFI values by 53% ($p < 0.0001$) and 56% ($p < 0.0001$), respectively. This result indicates that an additional N-truncation of $Aβ_{(1–42)}$ further increased the phagocytosis of PSPs. In contrast to the treatment of monocytes with soluble Aβ-peptides, pre-incubation with n-truncated $Aβ_{(2–40)}$ did not enhance phagocytosis more effectively than $Aβ_{(1–40)}$. Because undifferentiated monocytes are poor phagocytes, cytochalasin D only weakly reduced phagocytosis. Phagocytosis of pHrodo Green-labeled *E. coli,* which is only fluorescent at an acidic pH, revealed that cytochalasin D completely inhibited phagocytosis in our setting (Fig. 4F). Therefore, increased signal intensity after pretreatment with cytochalasin D and coincubation with

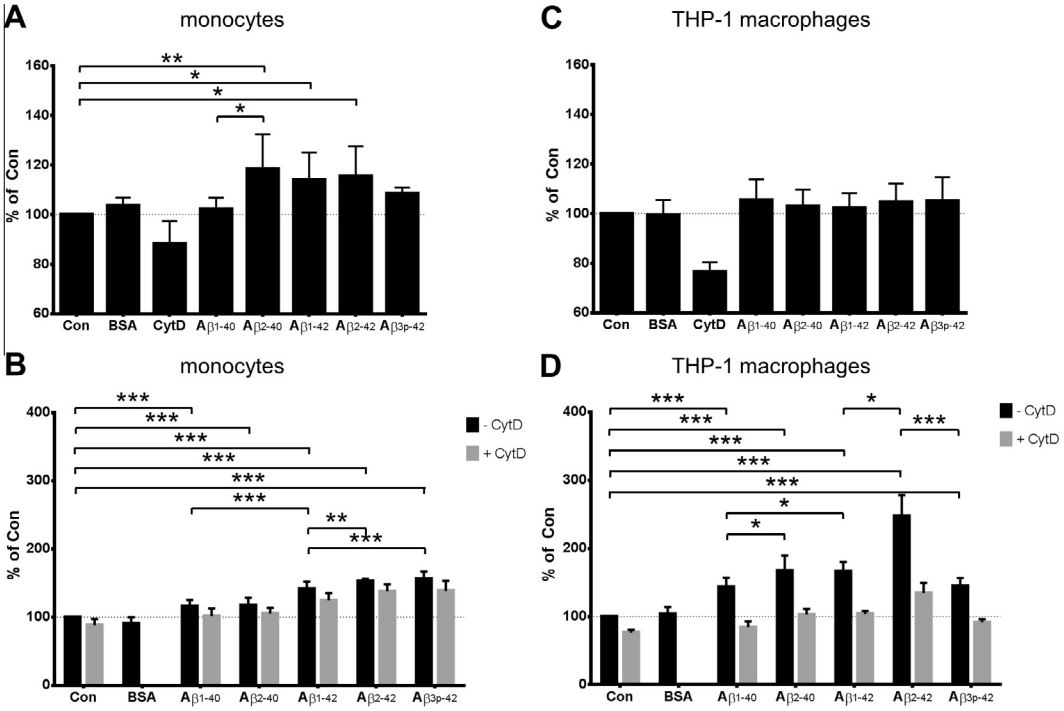

Fig. 1. Aβ-peptides facilitate phagocytosis by opsonizing prey. Aβ-peptide variants were added to the cell culture medium of freshly isolated monocytes (A) or differentiated THP-1 macrophages (C) at a concentration of 1 μg/mL. Alternatively, PSPs were pre-incubated with Aβ-peptide variants and added to cultures of monocytes (B) or THP-1 macrophages (D). The mean fluorescence intensity (MFI) of the phagocytes as a measure of the number of internalized fluorescent PSPs was quantified by flow cytometric analysis (black bars). Unspecific binding of the particles to the phagocytes was evaluated by adding 5 μM cytochalasin D to the medium (gray bars). The values of MFI are expressed relative to the untreated control cells (% of Con). The data represent the mean ± SD of five independent experiments. BSA = bovine serum albumin. CytD = cytochalasin D $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

permanently fluorescent prey indicated its binding to the phagocyte surface without internalization.

3.3. Differential effects of Aβ-peptides on phagocytosis of PSP were not caused by different binding affinities of the peptides for PSPs

To investigate whether the differential effects of the Aβ-peptides were due to different binding affinities for the PSPs, the amount of Aβ-peptide bound to the PSPs was quantified by immunostaining with the C- and N-terminal non-specific Aβ antibodies 6E10 and 4G8. Aβ-peptide-specific immunofluorescence detected on the PSPs did not differ between the Aβ-peptide species at coating concentrations of 1.0 mg/ml and 0.5 mg/ml (Fig. 2A and B). Because coating was performed at a concentration of 1.0 mg/ml, the observed effects on phagocytosis were not caused by the binding of different amounts of Aβ-peptides to the PSPs. Of note, compared to the other Aβ-peptide variants, more $Aβ_{(2–40)}$ bound to the PSP at the lower coating concentrations of 0.1 mg/ml and 0.05 mg/ml.

3.4. Phagocytosis of PSPs coated with $Aβ_{(x–42)}$ induced a proinflammatory state in monocytes

During the phagocytosis of Aβ-peptide-coated PSPs, the expression of several pro- and anti-inflammatory markers were examined by flow cytometry and ELISA. Coating of the PSPs with $Aβ_{(1–42)}$, $Aβ_{(2–40)}$, $Aβ_{(2–42)}$ and $Aβ_{(3p–42)}$ caused a 25–35% decrease in MSRI expression on the phagocytes compared to uncoated PSPs ($p < 0.05$). No significant effect was observed for $Aβ_{(1–40)}$ – or BSA-coated PSPs (Fig. 3A). Additionally, no significant alteration of the IL1 receptors or of CD206 was observed after coating the particles with Aβ peptides (Fig. 3B–D).

The IL-10 and TNFα levels were measured in cell culture supernatants after 72 h of phagocytosis of the Aβ-coated PSPs (Fig. 3E and F). The measurements were well above the limit of detection (1.56 pg/ml), and the coefficient of variation was below 25%. Compared to the phagocytosis of uncoated PSPs, the IL-10 levels were decreased by 20–30% only in monocytes treated with $Aβ_{(x–42)}$–coated PSPs ($p < 0.01$). Neither $Aβ_{(x–40)}$– nor BSA-coated PSPs changed the IL-10 expression in monocytes. The TNFα levels were only increased by coating the particles with $Aβ_{2–40}$.

The reduced expression of MSRI and the lower secretion of IL-10 indicate an induction of a proinflammatory polarization of monocytes during phagocytosis of $Aβ_{(x–42)}$ coated PSP.

3.5. In THP-1 macrophages, phagocytosis was induced by PSPs coated with Aβ-peptides but not by soluble Aβ-peptides in the culture medium

To assess the effects of Aβ-peptides on the phagocytosis of *in vitro*-differentiated phagocytes, THP-1 macrophages were analyzed in the assay described above. In contrast to human monocytes, the phagocytosis activity of THP-1 macrophages was not increased after adding soluble Aβ-peptides to the cell culture medium (Fig. 1C). Similar to freshly prepared monocytes, coating PSPs with all tested Aβ-peptides resulted in increased phagocytosis ($p < 0.0001$) (Fig. 1D). Among the untruncated $Aβ_{(1-x)}$ peptides, $Aβ_{(1–42)}$ was more active than $Aβ_{(1–40)}$ in stimulating the phagocytosis of PSPs ($p < 0.05$). Coating PSPs with N-terminally truncated $Aβ_{(2–40)}$ and $Aβ_{(2–42)}$ resulted in higher MFI values when compared to $Aβ_{(1–40)}$– and $Aβ_{(1–42)}$-coated PSPs, respectively ($p < 0.05$). The strongest induction of phagocytosis was observed with $Aβ_{(2–42)}$; compared to uncoated PSPs, the MFI values increased by 150% ($p < 0.0001$). Interestingly, $Aβ_{(3p–42)}$ was less effective than $Aβ_{(2–42)}$ in THP-1 macrophages, which is in contrast to our observations in primary human monocytes.

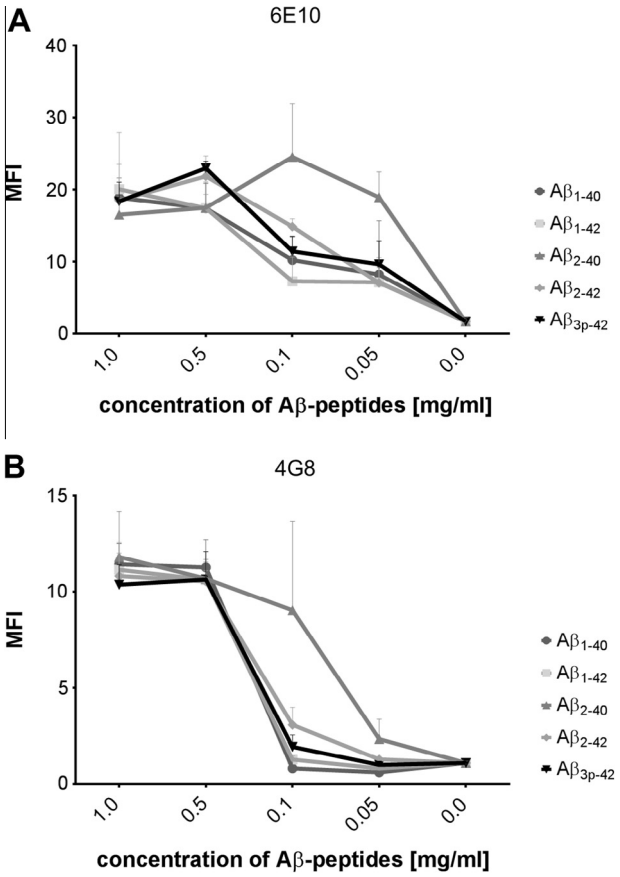

Fig. 2. Equal amounts of all Aβ-peptide variants on PSPs at a coating concentration of 1.0 mg/ml. PSPs were pre-incubated with Aβ-peptide variants at different concentrations. The amount of peptide bound to the PSP was measured by flow cytometry after staining with Aβ-specific antibodies 6E10 (A) or 4G8 (B) and is presented as the MFI of the particle. The data represent the mean ± SD of four independent experiments.

3.6. Phagocytosis of E. coli by THP-1 macrophages was enhanced by coating the bacteria with N-terminally truncated $A\beta_{(x-42)}$

Fluorescent, AF488-labeled *E. coli* were coated with the respective Aβ-peptides, and their phagocytosis was quantified by flow cytometry to investigate whether Aβ-peptides also act differentially on the phagocytosis of natural pathogens (Fig. 4G). Whereas no effect on the phagocytosis of *E. coli* was observed with the $A\beta_{(1-x)}$ isoforms, the phagocytosis of *E. coli* was strongly and exclusively enhanced by N-terminally truncated $A\beta_{(2-42)}$. A tendency to induce phagocytosis was also observed for $A\beta_{(3p-42)}$. This finding confirms that N-terminally truncated $A\beta_{(x-42)}$ also induces phagocytosis when bound to *E. coli*. As previously observed during the phagocytosis of PSPs, the opsonizing effect of $A\beta_{(3p-42)}$ was less pronounced in THP-macrophages than in primary human phagocytes.

3.7. $A\beta_{(x-42)}$-mediated phagocytosis of E. coli by primary human macrophages and porcine microglia

As differentiation and polarization have a great impact on the phagocytic activity of macrophages, primary human monocyte-derived GM-CSF- and M-CSF-elicited macrophages were compared. The differentiation and polarization of monocytes by GM-CSF and M-CSF were confirmed by phase contrast microscopy, iNOS immunofluorescence, flow cytometry and ELISA (Fig. 4A). GM-CSF-derived macrophages displayed higher expression of iNOS and CD206. The expression of MSRI, HLA-DR and CD14 was higher in M-CSF-elicited macrophages (Fig. 4B). Furthermore, the secretion of TNFα tended to be higher in GM-CSF-derived macrophages, whereas that of IL-10 was higher in M-CSF-derived macrophages (Fig. 4C). Therefore, GM-CSF-elicited macrophages shared several, but not all, of the features of M1 macrophages, whereas M-CSF-derived macrophages rather resembled M2 macrophages. Again, Aβ-peptides terminating at amino acid position 40 did not increase the uptake of AF488-labeled *E. coli*. Pre-incubation with n-truncated $A\beta_{(x-42)}$ increased the uptake of *E. coli* most effectively, independent of macrophage polarization. In GM-CSF-derived macrophages, coating with $A\beta_{(1-42)}$, $A\beta_{(2-42)}$ and $A\beta_{(3p-42)}$ resulted in 55–70% increases in the uptake of *E. coli* ($p < 0.01$). Most interestingly, $A\beta_{x-42}$ induced phagocytosis even more effectively than a commercial opsonizing (OpsR) reagent intended to facilitate the phagocytosis of *E. coli* (Fig. 4D). $A\beta_{5-42}$ also induced the phagocytosis of pHrhodo Green-labeled *E. coli*. However, this effect was weaker than that with $A\beta_{1-42}$ (Fig. 4F). Although a coating concentration of 1 mg/mL was chosen for the comparison of the Aβ peptide variants, a dose response analysis with $A\beta_{1-42}$ revealed 500 μg/mL to be the least effective coating concentration when applied in our paradigm (Supplementary Fig. 1).

In the M-CSF-derived macrophages, similar effects were obvious (Fig. 4E). N-terminally truncated $A\beta_{(3p-42)}$ stimulated the uptake of *E. coli* most efficiently. The MFI values increased by 67% ($p < 0.0001$). This effect was only slightly stronger after coating the *E. coli* with the opsonizing reagent (OpsR). $A\beta_{(1-42)}$ was again more effective than $A\beta_{(1-40)}$, which did not influence phagocytic activity ($p < 0.0001$).

The good correlation of fluorescent signal intensities between cultures with and without cytochalasin D ($r = 0.78$ for GM-CSF- and $r = 0.74$ for M-CSF-elicited macrophages, both $p < 0.001$) suggested that the Aβ peptides increased the phagocytosis of the prey by increasing its binding to the phagocytes (Supplementary Fig. 2). No such correlation was observed when using pHrodo Green-labeled particles, which were only fluorescent in acidic compartments ($r = 0.13$; $p = 0.41$).

Consistent with the published data, the total number of particles ingested by M-CSF-derived macrophages was twice as high as those taken up into GM-CSF-derived macrophages, independent of the coating of the particles (MFI: 47.13 ± 17.05 vs. 24.53 ± 5.37; $p < 0.0001$).

Primary porcine microglia were generated by separating loosely adherent cells from confluent mixed cortical cultures. Labeling by phagocytosis and CD14 revealed a purity of approximately 80%. When incubated with the Aβ peptide-coated AF488-labeled *E. coli*, the findings with macrophages could be reproduced. Again, the preincubation of *E. coli* with $A\beta_{1-42}$ and $A\beta_{3p-42}$ increased its uptake by phagocytes, with $A\beta_{3p-42}$ being more active than $A\beta_{1-42}$ (Fig. 5).

The results obtained in human macrophages and microglia confirmed that the coating of particles with N-terminally truncated $A\beta_{(x-42)}$ facilitates phagocytosis more effectively than coating with the other tested Aβ-peptides. Although M-CSF-derived macrophages showed higher phagocytic activity, the impact of Aβ-peptides was independent of the polarization of the macrophages.

4. Discussion

The present study provides evidence for an immunological function of Aβ-peptides as soluble factors and as opsonins, both of which promote the phagocytosis of pathogens. The effect of the Aβ-peptides depends on N- and C-terminal modifications. A proinflammatory phenotype is particularly induced by Aβ-peptides that terminate at alanine 42.

The phagocytosis of PSPs was facilitated by pre-incubation with all of the tested Aβ-peptide variants. Among them, $A\beta_{(x-42)}$ was

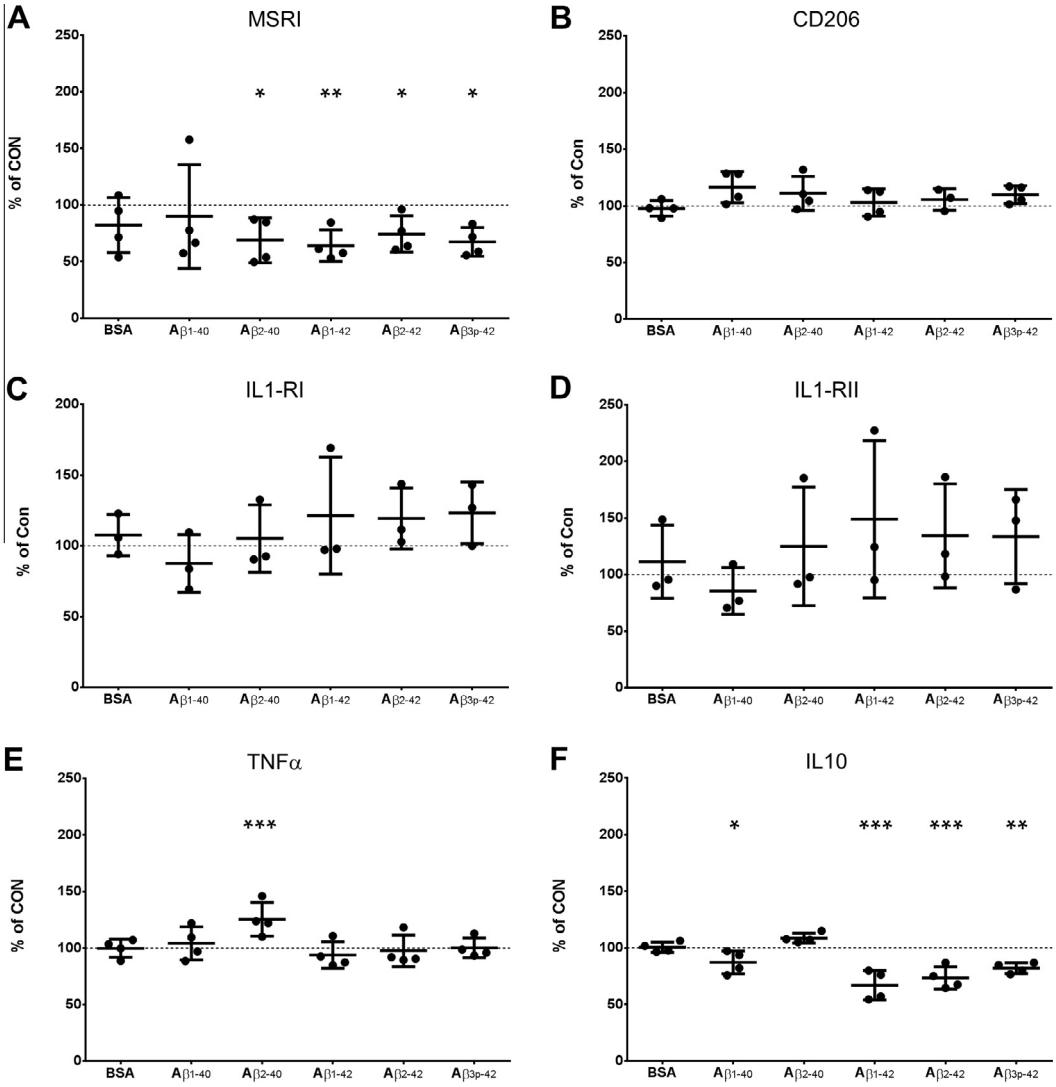

Fig. 3. Reduced expression of MSRI and reduced secretion of IL-10 after phagocytosis of PSPs coated with $A\beta_{(x-42)}$. Freshly isolated monocytes were cultivated with Aβ-peptide-coated PSPs for 72 h. Surface expression of the MSRI, CD206, IL1-RI and IL1-RII was quantified by flow cytometry (A–D). The IL-10 and TNFα levels in the conditioned media were determined by ELISA (E, F). The results are expressed in relation to untreated cells (% of Con). The data represent the mean ± SD of four independent experiments. Asterisks above the data indicate significance compared to the control. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

more efficient than $A\beta_{(x-40)}$. Similarly, an enhanced uptake of particles was previously observed in microglia after coating microspheres or yeast particles with $A\beta_{(1-42)}$ (Kopec and Carroll, 1998; Choucair-Jaafar et al., 2011). No such effect was reported after coating with $A\beta_{1-40}$ (Choucair-Jaafar et al., 2011). These reports and our data indicate that the C-terminus strongly impacts the phagocytosis-inducing effect of Aβ-peptides.

In primary monocytes and THP-1 macrophages, the phagocytosis of Aβ-coated particles was further increased by the N-terminal truncation of $A\beta_{(x-42)}$, i.e., $A\beta_{(2-42)}$ and $A\beta_{(3p-42)}$. As Aβ-peptides are highly hydrophobic, incubating particles with these peptides increases their hydrophobicity. Among the Aβ-peptides, those ending with alanine 42 are more hydrophobic than $A\beta_{(x-40)}$. N-truncation and pyroglutaminylation at amino acid residue 3 further enhance hydrophobicity due to the loss of charged groups (Pike et al., 1995; Schilling et al., 2006; Schlenzig et al., 2009; Meral and Urbanc, 2013). Hydrophobicity of the Aβ-peptides is also correlated with their aggregation propensity. Hence, the differences observed between the Aβ-peptide variants may also come from a heterogeneity in the size of Aβ-aggregates coated to the PSPs. However, oligomeric Aβ-peptides were reported to inhibit phagocytosis. (Pan et al., 2011). We therefor assume that the observed effect is not due to Aβ-peptide aggregation. In bacteria, increasing hydrophobicity facilitates their uptake by macrophages (Absolom, 1988; Tsuda et al., 2000; Nakano et al., 2008). Similarly, the phagocytosis-inducing effects of the different Aβ-peptide variants observed in our experiments correlated with their calculated hydrophobicity. Hydrophobicity is one of the hallmark characteristics of damage-associated molecular patterns (DAMP) (Seong and Matzinger, 2004). Cell damage or bacterial invasion is indicated whenever a hydrophobic domain of a protein is presented to the immune system. The receptors recognizing hydrophobic DAMPs are referred to as pattern recognition receptors (PRR), such as Toll-like or Scavenger receptors (Seong and Matzinger, 2004). For binding to PRR, hydrophobicity is the main predictive feature, and the binding interactions do not depend on the exact molecular configuration (Seong and Matzinger, 2004). The Aβ-peptides reportedly bind several PRRs, such as TLR2, TLR4 or CD36 (Salminen et al., 2009). Association with pathogens to increase hydrophobicity and improve the activation of PRR is a mechanism that has also been described for other proteins, such as surfactant or fibronectin (Seong and Matzinger, 2004). The concentration

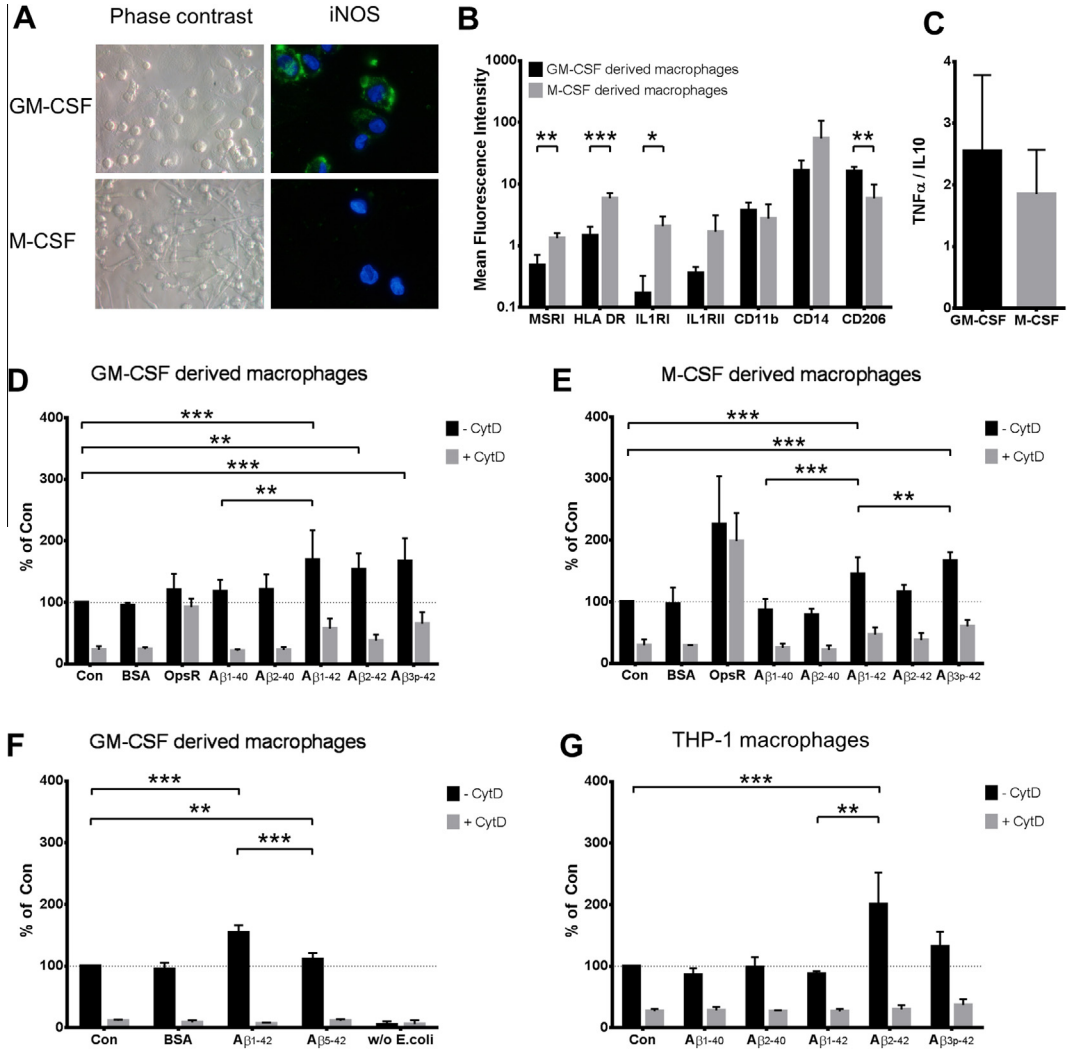

Fig. 4. $A\beta_{(x-42)}$ enhances the phagocytosis of *E. coli* by human macrophages. Characteristics of GM-CSF- and M-CSF-elicited macrophages are shown by the phase-contrast microscopic expression of inducible nitric oxide synthase (iNOS) (A), surface expression of MSRI, HLA DR, IL1-RI, IL1-RII CD11b, CD14 and CD206 (B) and secretion of TNFα relative to IL10 (C). Fluorescent AF-488 *E. coli* were coated with bovine serum albumin (BSA), opsonizing reagent (OpsR) or the respective Aβ-peptide variant and added to GM-CSF (D, F), M-CSF (E) or THP-1 (G) macrophage cultures. The number of internalized *E. coli* was evaluated by flow cytometry as indicated above (black bars). Unspecific binding of the particles to the phagocytes was evaluated by adding 5 μM cytochalasin D to the medium (gray bars). To control for the activity of cytochalasin D and to differentiate phagocytosis from unspecific binding, pHrodo Green-labeled *E. coli* were added to GM-CSF-elicited macrophages (F). The MFI values are expressed relative to the phagocytosis of uncoated *E. coli* (% of CON). The data represent the mean ± SD of four independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

used for coating the beads and *E. coli* particles in this study was approximately 1000-fold higher than that found in CSF or serum (Lewczuk et al., 2008; Lewczuk, 2009). Microdialysis experiments in patients with traumatic brain injury suggested $A\beta_{1-42}$ concentrations between 10 and 200 pg/mL in interstitial fluid (Tsitsopoulos and Marklund, 2013). However, the concentrations at the region of Aβ-peptide secretion are unknown and are most likely magnitudes higher than those measured at a steady state in the whole compartment. Through microdialysis, it was shown that stress or electrical activity could essentially increase the regional concentration of Aβ peptides (Cirrito et al., 2005; Kang et al., 2007). In the case of AD, it has been suggested that the reduced concentration of $A\beta_{1-42}$ in the CSF of AD patients was due to impaired transport of Aβ-peptides from the interstitial fluid into the CSF (Spies et al., 2012). The concentration of Aβ peptides in the interstitial fluid and especially in direct proximity to the plaques is therefore most likely increased in AD. Taken together, our data suggest that Aβ-peptides bound to particles facilitate phagocytosis. Opsonizing pathogens, in particular with N-terminally truncated $A\beta_{(x-42)}$, may therefore be a means to support phagocytosis in inflammatory processes.

In line with our study showing that soluble Aβ-peptides also induce the phagocytosis of fluorescent PSPs by monocytes, an enhanced uptake of particles was previously reported after priming microglia with soluble $A\beta_{(1-42)}$ (Kopec and Carroll, 1998). In addition to full-length $A\beta_{(1-42)}$, soluble N-terminally truncated $A\beta_{(2-40)}$ and $A\beta_{(2-42)}$ increased the uptake of PSP in our experiments. In contrast to phagocytosis of Aβ-coated particles, $A\beta_{(1-40)}$ and $A\beta_{(3p-42)}$ did not enhance phagocytosis when added to the cultures in a soluble form. This result indicates the involvement of different receptors for soluble Aβ-peptides than those that are bound to particles.

Our data show that the Aβ-peptide variants differ in their effect on mononuclear phagocytes, indicating distinct receptor binding profiles. This activity might explain why the strong phagocytosis-inducing effect of some Aβ-peptide variants (e.g., $A\beta_{(1-42)}$, $A\beta_{(2-40)}$ and $A\beta_{(2-42)}$) in monocytes was missing in differentiated macrophages that express a different repertoire of surface receptors.

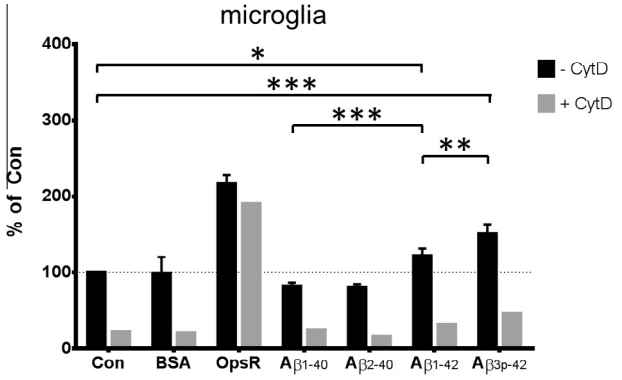

Fig. 5. $A\beta_{(x-42)}$ enhances the phagocytosis of *E. coli* by porcine microglia. Porcine microglia was challenged with Aβ peptide, bovine serum albumin (BSA) or opsonizing reagent (OpsR)-coated fluorescent AF488-labeled *E. coli* BioParticles (black bars). The addition of cytochalasin D (CytD) in one experiment revealed the unspecific binding of *E. coli* to the microglia (gray bars). The MFI values are expressed relative to the phagocytosis of uncoated *E. coli* (% of Con). The data represent means ± SDs of four independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Furthermore, the unresponsiveness of THP-1 macrophages toward $A\beta_{(3p-42)}$ may be due to the different receptor profile of this cell line. Therefore, it is essential to separately investigate the impact of each Aβ-peptide variant for each cell type. Distinct functions of Aβ-peptides are also reflected by the cell-specific secretion profiles of Aβ-peptide variants and by the different Aβ-peptide profiles in body fluids (Maler et al., 2007, 2009).

It was assumed that the binding of Aβ-peptides to microglia is deleterious, as this binding initiates a proinflammatory reaction leading to neuronal cell death (Rojo et al., 2008; Eikelenboom et al., 2011; Fricker et al., 2012; Neniskyte and Brown, 2013). In contrast, a physiological role for APP and Aβ-peptides in the immune system was suggested, as both induce chemotaxis in human monocytes and neutrophils (Tiffany et al., 2001; Kaneider et al., 2004). A relation between the immune system and Aβ-peptide metabolism is further supported by the observation of reduced Aβ-peptide levels in the CSF during the course of infectious diseases of the CNS, such as meningitis or borreliosis (Krut et al., 2013).

One source of the N-terminally modified Aβ-peptides detected in human plasma is the mononuclear phagocyte system (Maler et al., 2008). These cells are activated by phagocytosis, and they express higher levels of APP and release Aβ-peptides with increased proportions of N-terminally truncated $A\beta_{(x-40)}$ species (Ledoux, 1993; Spitzer et al., 2010). Remarkably, these N-terminally truncated $A\beta_{(x-40)}$ variants, when added in a soluble form to the cell culture medium, induced the phagocytosis of PSPs in primary human monocytes more effectively than other variants. Therefore, soluble Aβ-peptides, and especially N-terminally truncated variants secreted by mononuclear phagocytes, may act as auto- or paracrine pro-phagocytic factors employed by undifferentiated monocytes.

After the phagocytosis of Aβ-peptide-coated PSPs, we observed decreased levels of IL-10 secretion and MSRI expression by macrophages, indicating the beginning of a proinflammatory M1 polarization (Mantovani et al., 2004). Reduced secretion of IL-10 upon stimulation with $A\beta_{1-40}$ was previously observed in cultures of whole blood cells (Speciale et al., 2007). The missing increase in TNFα secretion and no obvious change in CD206 expression might indicate that the activation of macrophages by Aβ peptides was not clear-cut M1 polarization but was instead a mixed state with some preference for M1 characteristics. Although helpful as a basic model, dichotomous separation of M1 and M2 macrophages seemed to be an oversimplification. There has been increasing evidence that macrophages and microglia primarily express markers of both extremes and that each stimulus results in a specific activation state (Xue et al., 2014). Microglia in a Tg2576 AD mouse model were shown to express genes of classical activation (TNFα and NOS2), together with genes associated with an alternative activation (CD206, arginase I, chitinase-3-like-3) (Colton et al., 2006). This heterogeneity was also found in brain samples from AD patients (Sudduth et al., 2013). Interestingly, receptors binding Aβ-peptides such as TLR4, TLR2, RAGE or Scavenger receptors can induce pro- as well as antiinflammatory reactions of phagocytes for example by NFκB or MAPK signaling (Salminen et al., 2009; Canton et al., 2013; Zhang et al., 2014). In line with our data, Michelucci and colleagues found that the phagocytosis of $A\beta_{1-42}$ oligomeres induced markers that were associated with the M1 polarization of microglia (Michelucci et al., 2009). M1 polarization markers are especially induced by those Aβ-peptide variants that accumulate in Aβ-plaques during the course of AD (Guntert et al., 2006). Most likely as a consequence, microglia in the brains of AD patients shows signs of M1 polarization (Michelucci et al., 2009; Varnum and Ikezu, 2012; Sudduth et al., 2013).

Several studies have shown, in murine AD models, that inhibiting the proinflammatory M1 polarization of microglia with omega-3 fatty acids, IL10 or IL4 improved cognitive performance and reduced AD neuropathology (Varnum and Ikezu, 2012; Hjorth et al., 2013). The general proinflammatory M1 polarization of phagocytes is also found outside the CNS in AD patients (Varnum and Ikezu, 2012). Proinflammatory cytokines, which induce M1 polarization, seem to inhibit the clearance of Aβ by macrophages (Town et al., 2005; Yamamoto, 2008). This activity might be explained by the observed lower phagocytosis rate of M1 compared to M2 macrophages. However, we found that the phagocytosis-inducing effect of Aβ-peptides was similar in M1 and M2 macrophages. This result indicates that opsonizing pathogens with Aβ-peptides improves phagocytosis, but a concurrent differentiation in the direction of M1 macrophages may ameliorate this effect.

In the pathogenesis of AD, Aβ peptides that terminate after AA 42, particularly the N-terminally truncated and pyroglutaminylated variant $A\beta_{3p-42}$, have been considered important pathogenic agents (Portelius et al., 2010; Wittnam et al., 2012). They are the main components of neuritic plaques, and the toxicity of $A\beta_{1-42}$ and, even more significantly, $A\beta_{3p-42}$ toward neurons has been well established (Wirths et al., 2009; Portelius et al., 2010; Becker et al., 2013). Consequently, the inhibition of glutaminyl cyclase, which catalyzes the pyroglutaminylation step, is considered a potential treatment for AD (Alexandru et al., 2011).

Another approach to stopping AD progression that is currently under clinical investigation is the inhibition of BACE1. Interestingly, inhibitors of BACE1 reduced $A\beta_{1-x}$ species, with a relative increase in the N-terminally truncated Aβ peptide variants, such as $A\beta_{5-x}$ (Takeda et al., 2004; Portelius et al., 2011; Mattsson et al., 2012). In our experiments, we found $A\beta_{5-42}$ to support the phagocytosis of *E. coli*. There has been growing evidence that the secretion of N-terminally truncated Aβ-peptides is not dependent on BACE1. An enzyme suggested to be involved in this process is meprin-β (Bien et al., 2012). Meprin-β is also expressed by mononuclear phagocytes, and meprin deficiency has been associated with a dysfunction of monocytes, leading to reduced immunoresponsiveness (Crisman, 2004; Sun et al., 2009).

Several other lines of evidence support the idea of chronic systemic inflammation as the driving force in plaque deposition, linking it with immunosenescence and a consequently lower immune responsiveness in AD (Malavolta et al., 2013). For example, several pro-inflammatory cytokines, such as TNFα, IL1β and IL6, are increased in AD, natural killer cells seem to be normal in frequency but defective in function and there is a general decline in T-cell

responsiveness (Solerte et al., 2000; Swardfager et al., 2010; Jadidi-Niaragh et al., 2012; Monsonego et al., 2013). Cashman et al. suggested that Aβ-aggregation in AD is a result of impaired innate immunity together with defective Aβ phagocytosis (Cashman et al., 2008). Furthermore, monocytes from patients with AD are deficient in PRR expression, and mitogen-stimulated whole-blood cell cultures from AD patients secrete lower levels of proinflammatory cytokines (Richartz et al., 2005; Fiala et al., 2007).

We propose that the production and phagocytosis of Aβ peptides is, as with reactive oxygen species, a tightly regulated defense mechanism of the immune system in the blood and brain. Disturbances of this homeostasis might lead to amyloid deposition, neurodegeneration and finally dementia. Currently, one can speculate whether the defective clearance of Aβ-peptides in patients with AD is the result of reduced immune responsiveness and that this reduced immune responsiveness may result from a primary energy toward Aβ-peptides.

5. Conclusion

The present study indicates that soluble and surface-bound Aβ-peptides facilitate phagocytosis depending on the length of their N- and C-termini and that they induce a proinflammatory polarization of the phagocytes. On one hand, these findings indicate a physiological function of Aβ-peptides. On the other hand, an Aβ-driven proinflammatory M1 polarization impairing phagocytosis may provide a model for self-sustained plaque deposition.

6. Competing interests

The authors declare that they have no competing interests.

7. Authors' contributions

J.M.M., M.H. and P.S. designed the study. M.C., J.M.M. and P.S. conducted the study and prepared the manuscript. M.H. provided expertise in interpreting the results of the flow cytometry-based phagocytosis assay. M.R. isolated the porcine microglia. J.T.O. and J.K. reviewed the manuscript extensively and provided constructive comments to improve the quality of the manuscript. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2014.05.003.

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